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Modulation of hepatocyte growth factor induction in human skin fibroblasts by retinoic acid

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Abstract

Topical treatment of skin with all-*trans*-retinoic acid (ATRA), the major biologically active form of vitamin A, results in hyperproliferation of basal keratinocytes, leading to an accelerated turnover of epidermis cells and thickening of the epidermis, probably via induction of production of paracrine growth factors for keratinocytes in epidermal suprabasal keratinocytes and/or dermal fibroblasts. Since hepatocyte growth factor (HGF) is a factor mitogenic to epidermal keratinocytes secreted from dermal fibroblasts, the effect of ATRA on basal and induced HGF production in human dermal fibroblasts in culture was examined. ATRA alone did not induce HGF production, but it significantly enhanced HGF production induced by the cAMP-elevating agent cholera toxin or the membrane-permeable cAMP analog 8-bromo-cAMP. Cholera toxin-induced activation of cAMP responsive element (CRE)-binding protein (CREB) was enhanced by pretreating cells with ATRA for 24 h. In contrast, HGF production induced by epidermal growth factor (EGF) or phorbol 12-myristate 13-acetate (PMA) was potently inhibited by ATRA. These modulatory effects of ATRA were different from the effects of transforming growth factor-β1 (TGF-β) and dexamethasone, both of which inhibited HGF production induced by all of the four inducers. Up-regulation of HGF gene expression by cholera toxin and EGF was also enhanced and inhibited, respectively, by ATRA. Both 9-*cis*-retinoic acid (9-*cis*-RA) and 13-*cis*-retinoic acid (13-*cis*-RA), which are stereo-isomers of ATRA, showed a modulatory effect on HGF induction similar to that of ATRA. These results suggest that ATRA augments the induction of HGF production caused by increased intracellular cAMP.

Keywords: Hepatocyte growth factor; All-*trans*-retinoic acid; cAMP; Induction; Skin fibroblast; Keratinocyte

1. Introduction

All-*trans*-retinoic acid (ATRA) is the major biologically active form of vitamin A [1]. When applied topically on normal adult human and mouse skin, ATRA stimulates keratinocyte proliferation, resulting in an increase in the number of epidermal cell layers and thus an increase in epidermal thickness [2]. ATRA treatment also decreases the cohesiveness of the stratum corneum, impairing the adult skin barrier and increasing trans-epidermal water loss [3]. Although the molecular events underlying the actions of ATRA in skin have not been elucidated, it has been suggested that ATRA stimulates proliferation of keratinocytes indirectly by inducing the production of paracrine growth factors for keratinocytes from the epidermis and/or the dermis. Recent studies have shown that the expression of heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) in suprabasal keratinocytes and the expression of keratinocyte growth factor (KGF) in cultured gingival fibroblasts are stimulated by ATRA [4–7].

Hepatocyte growth factor (HGF), also known as scatter factor, is a factor mitogenic to keratinocytes produced in

Abbreviations: AP-1, activator protein-1; ATRA, all-*trans*-retinoic acid; 9-*cis*-RA, 9-*cis*-retinoic acid; 13-*cis*-RA, 13-*cis*-retinoic acid; CBP, CREB-binding protein; CRE, cAMP responsive element; CREB, CRE-binding protein; DMEM, Dulbecco's modification of Eagle's minimum essential medium; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; HGF, hepatocyte growth factor; KGF, keratinocyte growth factor; PMA, phorbol 12-myristate 13-acetate; RAR, retinoic acid receptor; RXR, retinoid X receptor; TGF-β, transforming growth factor-β

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various fibroblasts, including human skin fibroblasts [8–12]. HGF stimulates the proliferation of human keratinocytes in a medium having a physiologic calcium concentration as potent as KGF [10]. HGF was initially isolated as a potent mitogenic factor for adult rat hepatocytes in primary culture [13–17], but HGF has been shown to act on many kinds of epithelial cells other than hepatocytes and on other types of cells, and it has been shown to have multiple activities, including mitogenic, motogenic, morphogenic, and tumor-inhibiting activities [18–22]. HGF is induced by the activation of protein kinase A and protein kinase C-mediated pathways [12,23,24]. Its production is also stimulated by interleukin-1, tumor necrosis factor-α, interferon-γ, estrogen, ascorbic acid, okadaic acid, norepinephrine, a scatter factor-inducing factor, and growth factors such as EGF and basic fibroblast growth factor [25–32].

The present study was designed to determine effects of ATRA on HGF production and HGF induction in human skin fibroblasts in culture. We found that induction of HGF by various inducers was differently regulated by ATRA, although ATRA alone did not significantly affect HGF production: cholera toxin- and 8-bromo-cAMP-induced HGF production was enhanced by ATRA, while EGF- and phorbol 12-myristate 13-acetate (PMA)-induced HGF production was inhibited by ATRA.

2. Materials and methods

2.1. Reagents

Dulbecco's modification of Eagle's minimum essential medium (DMEM) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). ATRA, 9-*cis*-retinoic acid (9-*cis*-RA), 13-*cis*-retinoic acid (13-*cis*-RA), 8-bromo-cAMP, cholera toxin, and PMA were obtained from Sigma Chemical Co. (St. Louis, MO). Mouse EGF was from BD Biosciences (San Jose, CA). Anti-cAMP responsive element-binding protein (CREB)-binding protein (CBP) antibody and anti-p300 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). [α-^{32}P]dCTP (~110 TBq/mmol) was from Amersham Biosciences (Little Chalfont, England). Human HGF cDNA (*Bam*HI/*Kpn*I fragment, 2.2 kbp) was derived from plasmids originally obtained from Dr. Naomi Kitamura (Tokyo Institute of Technology, Yokohama, Japan). Other reagents were obtained from previously reported sources [24].

2.2. Cell culture

Normal human skin fibroblasts isolated from 200 individual neonatal donors (Cell Systems, Kirkland, WA) were used between the seventh and tenth passages. The cells were grown as monolayers in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, as described previously [12].

2.3. Determination of HGF levels in conditioned media

Human skin fibroblasts, trypsinized and suspended in the medium described in the previous section, were seeded in 96-well plates (Nunc, Roskilde, Denmark) at a density of 1.8×10^4 cells/cm^2 (0.17 ml/well). After reaching confluence, the medium was replaced with the same fresh medium containing ATRA, other retinoic acids and HGF inducers. The conditioned medium was collected after incubating the cells for 72 h, unless stated otherwise, and was frozen at −30 °C for a human HGF ELISA. The sandwich ELISA for human HGF was performed at room temperature as described previously [33], with a slight modification [26], except that biotinylated goat anti-human HGF antibody (R&D Systems, Minneapolis, MN) and horseradish peroxidase-streptavidin conjugate (Zymed Laboratories, San Francisco, CA) were used as a detection antibody and a horseradish peroxidase conjugate, respectively. HGF levels were expressed as ng/mg cellular protein or ng/ml as described previously [24]. Cellular protein was determined as described previously [24].

2.4. Northern blot analysis

The medium of confluent human skin fibroblasts grown in 9-cm dishes (Nunc) was replaced with the same fresh medium containing ATRA and HGF inducers. After incubating for 40 h, total RNA was isolated from the cells using RNA-Bee (TEL-TEST, Friendswood, TX). Northern blotting was performed as described previously [24]. Briefly, total RNA (10 μg) was denatured with 2.2 M formaldehyde and 50% formamide, mixed with ethidium bromide (50 μg/ml), and fractionated on 1% agarose gels containing 2.2 M formaldehyde. The gel was photographed by ultraviolet illumination. The RNA was then transferred from the gel to a Biodyne nylon membrane. The membrane-bound RNA was hybridized to a ^{32}P-labeled human HGF cDNA probe. After being washed, the membrane was exposed to an imaging plate at room temperature, and the plate was analyzed using a Bio-imaging analyzer, BAS-2000 (Fuji Photo Film Co., Tokyo, Japan). The signal intensity of the 6.8-kb HGF mRNA band was normalized to the fluorescence intensity of the 28S rRNA band and expressed as fold-change relative to the control cultures, which were incubated in the medium alone. The human HGF cDNA fragment was labeled with [α-^{32}P]dCTP by the megaprime DNA labeling system (Amersham Biosciences) according to the manufacturer's instructions.

2.5. Transcription factor ELISAs

The medium of confluent human skin fibroblasts grown in 9-cm dishes (Nunc) was replaced with the

same fresh medium containing ATRA and incubated for 2 or 24 h before adding cholera toxin. After incubating for 1.5 h with cholera toxin plus ATRA, nuclear extracts of cells were prepared according to protocol instructions (Active Motif, Carlsbad, CA). Activation of CREB in nuclear extracts of cells was measured by the use of the TransAM pCREB kits (Active Motif) according to the manufacturer's instructions. Phosphorylated CREB bound specifically to a fixed oligonucleotide containing the cAMP-responsive element (CRE) 5′-TGACGTCA-3′, and was detected through use of an anti-phosphorylated CREB antibody.

2.6. Western blot analysis

The medium of confluent human skin fibroblasts grown in six-well plates (Nunc) was replaced with the same fresh medium containing ATRA or ATRA plus cholera toxin, and the cultures were incubated for 24 h. Cells were then washed once with ice-cold PBS, scraped into PBS, and washed three times more with PBS. Cells were lysed by adding 100 μl of SDS sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% Bromophenol Blue). Lysates were boiled for 10 min, briefly sonicated, and centrifuged. Protein in extracts was determined by a modification of the method of Lowry et al. [34]. Western blotting was performed as described previously [35]. Briefly, protein aliquots (10 μg) were separated by 6% SDS-PAGE, transferred electrophoretically to Immobilon-P transfer membranes (Millipore, Billerica, MA). Ponceau S staining was performed in order to ensure equivalent gel loading. Membranes were probed with anti-CBP antibody or anti-p300 antibody. Membranes were then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences) and detected with the ECL Plus Western blotting detection reagents (Amersham Biosciences). Densitometric analysis of the bands was performed using UMAX PowerLook II flat-bed scanner (UMAX Data Systems, Taipei, Taiwan) and Intelligent Quantifier software (Bio Image Systems, Ann Arbor, MI).

2.7. Data analysis

All results are expressed as means and S.E. of several independent experiments. The data were analyzed by Dunnett's test, Bonferroni's test or paired Student's *t*-test. *P* values less than 5% were regarded as significant.

3. Results

Human skin fibroblasts were incubated for 72 h with various doses of ATRA, and the amounts of HGF secreted into the media were measured by an HGF ELISA. ATRA alone did not increase HGF production at concentrations of 0.01–10 μM (Table 1). HGF production in human skin fibroblasts is markedly induced by cholera toxin, 8-bromo-cAMP, EGF, and PMA [12,24,32]. The effect of ATRA on HGF production stimulated by optimal concentrations of these inducers was next examined. Cholera toxin- and 8-bromo-cAMP-induced HGF production was significantly enhanced by 0.1–10 μM ATRA (Table 1). The greatest enhancement of cholera toxin-induced HGF production and that of 8-bromo-cAMP-induced HGF production by ATRA were 73% and 66%, respectively. Cholera toxin- and 8-bromo-cAMP-induced HGF production, expressed as ng/ml instead of ng/mg cellular protein, was also augmented by the same concentrations of ATRA (data not shown). In contrast, EGF-induced HGF production and PMA-induced HGF production were dose-dependently inhibited by 1–10 and 0.01–10 μM ATRA, respectively (Table 1). Ten micromolar ATRA inhibited EGF- and PMA-induced HGF production by 74% and 87%, respectively. ATRA did not significantly affect protein levels of EGF- or PMA-treated cell cultures (data not shown).

Fig. 1 shows time courses of the effects of ATRA on cholera toxin- and EGF-induced HGF production. The inhibiting activity of ATRA toward EGF-induced HGF production was detected as early as 24 h after the start of incubation, while significant enhancement of cholera toxin-induced HGF production by ATRA was not detected until 48 h after the start of incubation.

Table 1
Effect of ATRA on basal and induced HGF production

HGF inducer	HGF secreted (ng/mg cellular protein)				
	ATRA (μM)				
	0	0.01	0.1	1	10
None	0.7±0.2	0.7±0.2	1.0±0.3	0.8±0.2	0.4±0.2
Cholera toxin	44.0±3.7	49.1±3.1	64.2±3.3**	76.3±4.8**	65.5±2.8**
8-Bromo-cAMP	97.4±4.8	138.6±8.7*	162.1±9.6**	158.2±10.9**	139.1±7.6*
EGF	25.5±2.0	22.0±0.7	18.9±0.6	12.1±0.7*	6.6±0.7**
PMA	11.1±0.5	5.3±0.8**	3.9±1.1**	2.4±0.8**	1.4±0.7**

Confluent human skin fibroblasts were incubated for 72 h with the indicated concentrations of ATRA in the presence or absence of 10 pM cholera toxin, 1 mM 8-bromo-cAMP, 10 ng/ml of EGF or 30 nM PMA. The HGF secreted into the medium was measured by an ELISA. The data are mean±S.E. of four independent experiments. Values that are significantly different from those of the respective control (medium or inducer alone) are indicated by *$P<0.05$, **$P<0.01$ (Dunnett's test).

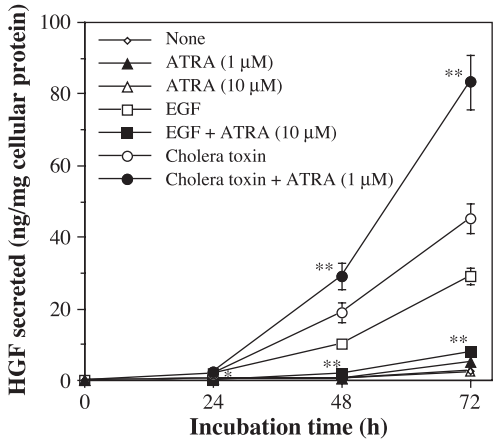

Fig. 1. Time courses of the effects of ATRA on basal and cholera toxin- and EGF-induced HGF production. Confluent human skin fibroblasts were incubated for the indicated periods with various additives. The concentrations of cholera toxin and EGF were 10 pM and 10 ng/ml, respectively. The HGF secreted into the medium was measured by an ELISA. The data are mean±S.E. of four independent experiments. Where S.E. bars are not shown, the S.E. was smaller than the symbol. Values that are significantly different from those of the respective control (inducer alone) are indicated by $*P<0.05$, $**P<0.01$ (Student's *t*-test). ATRA (1 and 10 μM) alone did not significantly influence HGF production at any incubation time (Dunnett's test).

The effects of ATRA on basal and induced HGF gene expressions determined 40 h after the addition of ATRA are shown in Fig. 2. ATRA did not significantly increase the basal HGF mRNA level but increased the HGF mRNA level up-regulated by cholera toxin. In contrast, the induced HGF mRNA expression in EGF-treated cells was decreased by ATRA.

Transforming growth factor-β (TGF-β) and glucocorticoids are inhibitors of HGF production in various cells, including human dermal fibroblasts [12,36–38]. The effect of ATRA on HGF production stimulated by various HGF inducers was compared with the effects of TGF-β1 and dexamethasone. The inhibitory effect of ATRA on PMA-induced HGF production was comparable to the effects of TGF-β1 and dexamethasone, whereas the inhibitory effect of ATRA on EGF-induced HGF production was less potent than the effects of TGF-β1 and dexamethasone (Fig. 3). Cholera toxin- and 8-bromo-cAMP-induced HGF production was strongly inhibited by TGF-β1 and dexamethasone but enhanced by ATRA as described above (Fig. 3).

Biological effects of retinoids are mediated by two families of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which are ligand-dependent transcription factors [39]. Each receptor family consists of three members, α, β and γ. The two receptor families display distinct ligand specificities. The RAR family is activated by both ATRA and 9-*cis*-RA, whereas the RXR family is activated by 9-*cis*-RA. In the form of RXR/RAR heterodimers or RXR/RXR homodimers, the receptors act through binding to retinoic acid response elements present in the transcriptional regulatory region (promoter) of target genes. Another stereo-isomer of ATRA, 13-*cis*-RA has little or no binding properties to RARs [40], but it is converted to ATRA in the cells [41]. The effects of 9-*cis*-RA and 13-*cis*-RA on basal and induced HGF production were determined. As shown in Fig. 4, both retinoic acids, like ATRA, enhanced cholera toxin- and 8-bromo-cAMP-induced HGF production and inhibited EGF- and PMA-induced HGF production. The three retinoic acids showed similar dose–response curves for cholera toxin-, 8-bromo-cAMP-, and PMA-induced HGF production. The inhibitory effect of 13-*cis*-RA on EGF-induced HGF production was less potent than the inhibitory effects of the other two retinoic acids. Neither 9-*cis*-RA nor 13-*cis*-RA alone affected HGF production (data not shown).

The mechanisms underlying the synergistic induction of HGF by ATRA and cholera toxin were next examined. The transcription factor CREB is important in the activation of transcription of many cAMP-responsive genes. Phosphorylation of Ser 133 is required for CREB-mediated transcription [42] and the cofactors CBP and p300 specifically bind to phosphorylated CREB to enhance transcriptional activity [43]. Thus, we determined an effect of ATRA on the

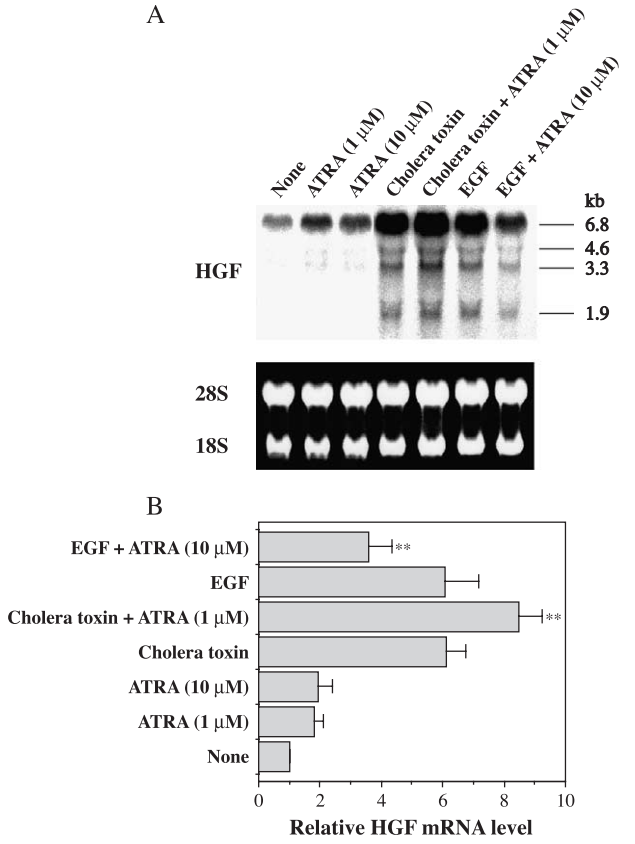

Fig. 2. Effect of ATRA on gene expression of HGF up-regulated by cholera toxin or EGF. Confluent human skin fibroblasts were incubated for 40 h with or without ATRA in the presence or absence of 10 pM cholera toxin or 10 ng/ml of EGF. Total RNA was isolated and Northern-blotted using a ^{32}P-labeled cDNA probe for human HGF. Autoradiographs and fluorescence photographs (A) are representative of six independent experiments. The data (B) are means of six independent experiments. Bars indicates S.E. Values that are significantly different from the respective control (inducer alone) are indicated by $**P<0.01$ (Student's *t*-test). ATRA (1 and 10 μM) alone did not significantly increase the level of HGF mRNA (Dunnett's test).

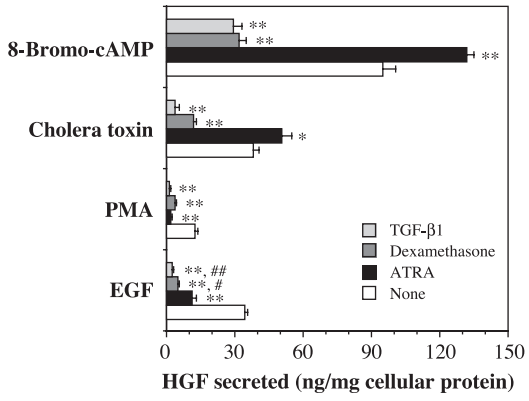

Fig. 3. Comparison of the inhibitory effect of ATRA on HGF induction with the inhibitory effects of TGF-β1 and dexamethasone. Confluent human skin fibroblasts were incubated for 72 h with 10 μM ATRA, 10 μM dexamethasone and 1 ng/ml of TGF-β1 in the presence or absence of various HGF inducers (10 ng/ml of EGF, 30 nM PMA, 10 pM cholera toxin and 1 mM 8-bromo-cAMP). The HGF secreted into the medium was measured by an ELISA. The data are means of three independent experiments. Bars indicate S.E. Values that are significantly different from those of the respective control (inducer alone) are indicated by $*P<0.05$, $**P<0.01$ (Dunnett's test). $^{\#}P<0.05$, $^{\#\#}P<0.01$, as compared with EGF plus ATRA or PMA plus ATRA (Bonferroni's test).

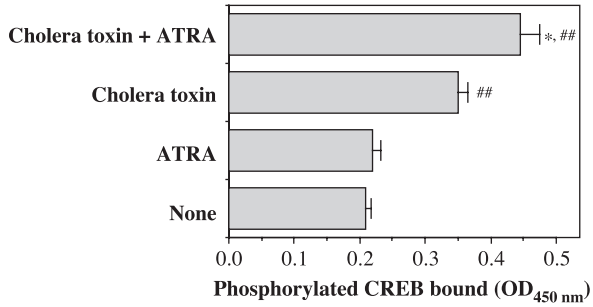

Fig. 5. Enhancement of cholera toxin-induced CREB activation by prolonged pretreatment with ATRA. Confluent human skin fibroblasts were preincubated for 24 h with or without 1 μM ATRA and then incubated with or without cholera toxin (10 pM) for 1.5 h. Equal amounts of nuclear extracts (1.9 μg) were used to quantify phosphorylated CREB by an ELISA. The data are means of three independent experiments. Bars indicates S.E. Values that are significantly different from the respective control (none or cholera toxin alone) are indicated by $*P<0.05$ (Bonferroni's test). $^{\#\#}P<0.01$, as compared with none (Bonferroni's test).

activation of CREB and levels of CBP and p300 proteins in cells treated with or without cholera toxin. Activation of CREB, measured by an ELISA-based method, in cholera toxin-treated cells did not change by 2-h pretreatment with

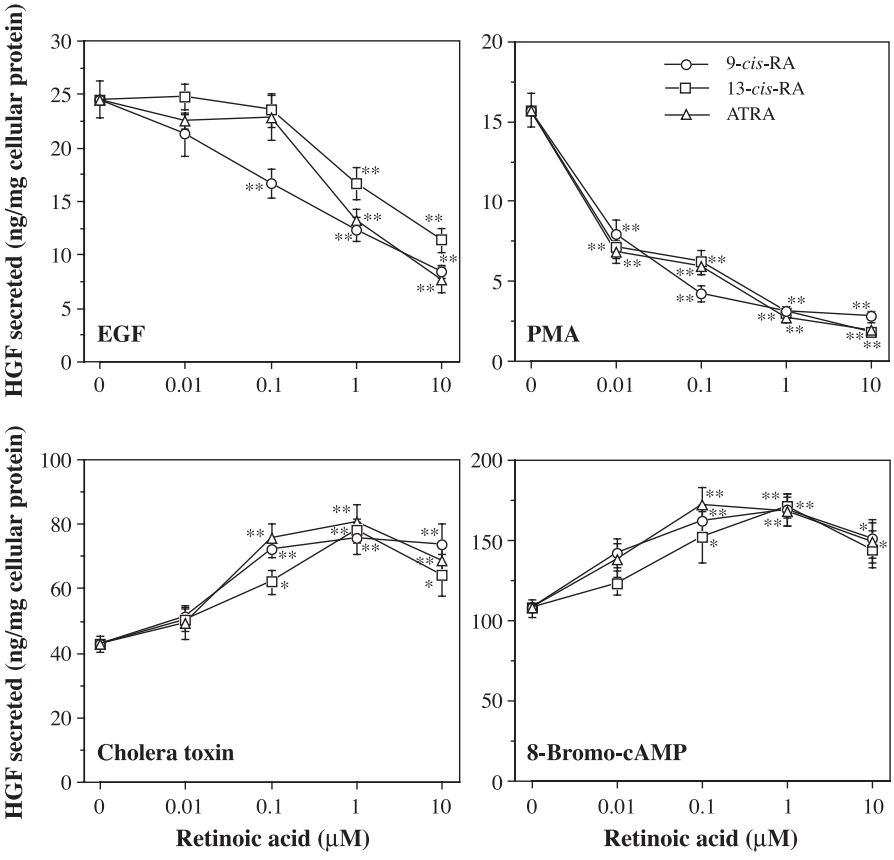

Fig. 4. Effects of 9-*cis*-RA and 13-*cis*-RA on HGF production stimulated by various inducers. Confluent human skin fibroblasts were incubated for 72 h with the indicated concentrations of 9-*cis*-RA, 13-*cis*-RA and ATRA in the presence of various HGF inducers (10 ng/ml of EGF, 30 nM PMA, 10 pM cholera toxin and 1 mM 8-bromo-cAMP). The HGF secreted into the medium was measured by an ELISA. The data are mean±S.E. of four independent experiments. Where S.E. bars are not shown, the S.E. was smaller than the symbol. Values that are significantly different from those of the inducers alone are indicated by $*P<0.05$, $**P<0.01$ (Dunnett's test).

ATRA (data not shown). However, 24-h pretreatment with ATRA significantly enhanced the activation of CREB in cells incubated for 1.5 h with cholera toxin but not in cells incubated in medium alone (Fig. 5). Incubation with CREB wild-type competitor oligonucleotide (5′-AGAGATTGCCT-GACGTCAGAGAGCTAG-3′) reduced phosphorylated CREB binding by over 90%, while incubation with CREB mutated oligonucleotide (5′-AGAGATTGCCGACCATAG-GAGAGCTAG-3′) had no effect (data not shown). The amounts of neither CBP protein nor p300 protein increased after 24-h treatment with ATRA or ATRA plus cholera toxin (data not shown).

4. Discussion

Recent studies have shown that induction of HB-EGF is involved in epidermal hyperplasia generated by topical application of ATRA: ATRA induces production of HB-EGF in suprabasal keratinocytes, which in turn stimulates basal cell growth via paracrine/juxtacrine mechanisms [4–6]. RXRα/RARγ heterodimers play a key role in ATRA-induced HB-EGF expression in suprabasal cells [6]. However, HB-EGF may not be the sole signaling molecule involved in ATRA-induced proliferation, since ATRA does not increase HB-EGF expression in RARγ-null mice, whereas it still exerts some proliferative effect on their epidermis [6]. Growth factors for keratinocytes secreted from dermal fibroblasts and induced by ATRA are possible paracrine factors. A recent study has shown that expression of KGF is stimulated by ATRA in cultured gingival fibroblasts [7]. HGF is a mitogen for keratinocytes secreted from human dermal fibroblasts [8–10,12], but its production was not stimulated by ATRA (Table 1), although RARα and RARγ, but not RARβ, are expressed in human dermal fibroblasts and the expression of RARβ is induced by ATRA [44]. However, ATRA enhanced HGF production induced by cholera toxin, which increases intracellular cAMP level [24], and that induced by the membrane-permeable cAMP analog 8-bromo-cAMP. Recent study of Sato et al. [45] showed that cell–cell interactions of human epidermal keratinocytes and dermal fibroblasts in co-culture increase the production of prostaglandin E_2 by a mechanism in which the activity of cyclooxygenase-2 in fibroblasts is induced by the keratinocyte-derived precursor of interleukin-1α in a paracrine manner. Consequently, an appreciable amount of prostaglandin E_2 (approximately 30 ng/g of wet tissue) is present in normal human skin [46]. Since prostaglandin E_2 induces HGF production in human skin fibroblasts by increasing the level of intracellular cAMP [24], ATRA might augment HGF production induced by prostaglandin E_2 in human skin.

Several other instances of synergism between ATRA and cAMP are known: both agents synergistically induce the expression of urokinase-type plasminogen activator, alkaline phosphatase, and matrix metalloproteinase-2 [47–49]. However, the molecular mechanisms responsible for these synergistic effects are not fully understood. We detected an enhancement of cholera toxin-induced activation of CREB in cells pretreated with ATRA for 24 h as potential mechanisms underlying the synergistic induction of HGF by ATRA and cholera toxin. Cholera toxin-induced activation of CREB, however, was not increased by 2-h pretreatment with ATRA. Since ATRA treatment for 15 or 24 h increases protein kinase A activity in some cell lines [50,51], the enhanced activity of protein kinase A may be involved in the augmented activation of CREB. It was recently shown that protein expression of both CBP and p300 was induced in HSC-3 human oral cancer cell line treated with 9-*cis*-RA for 48 h and in ATRA-resistant MCF-7 variant cells treated with ATRA for 24 h when RARβ2 gene was transfected [52,53]. However, we were unable to detect any increase in the amounts of CBP and p300 in human dermal fibroblasts treated with ATRA for 24 h. We do not know the exact reason for this discrepancy at present.

A recent study of Jiang et al. [54] showed peroxisome proliferator-activated receptor γ (PPARγ)-mediated transcriptional up-regulation of the mouse HGF gene. The mouse HGF gene promoter activity in mouse 3T3-L1 fibroblasts was stimulated by PPARγ transfected plus its ligand, 15-deoxy-prostaglandin J_2. It is believed that RXRα is the functional partner of PPARγ through heterodimer formation. A combination of 9-*cis*-RA plus its receptor, RXRα, transfected also stimulated HGF promoter activity. HGF production in human skin fibroblasts, however, was not stimulated by 9-*cis*-RA alone, although RXRα and RXRβ, but not RXRγ, are expressed in human skin fibroblasts [55].

In contrast to cholera toxin- and 8-bromo-cAMP-induced HGF production, EGF- and PMA-induced HGF production was inhibited by ATRA. Activator protein-1 (AP-1), which is a complex composed of Jun homodimers and Jun/Fos heterodimers and is induced by growth factors and tumor promoters [56], could be involved in EGF- and PMA-induced HGF production. It is known that ATRA shows anti-AP-1 activity through repression of AP-1 transcriptional activity, initiated by the tumor promoter PMA [57]. This AP-1 activity inhibition is not related to direct interactions between retinoid receptors and retinoic acid responsive elements present in the transcriptional regulatory regions (promoters) of AP-1 genes. It might be associated with direct protein–protein interactions between retinoid receptors and components of AP-1, such as c-Jun and c-Fos, resulting in the inhibition of AP-1 DNA binding [58]. Alternatively, it might be associated with competition for binding to the limited amount of the common coactivator CBP/p300, which is required for the transcriptional activating effect of AP-1 and nuclear receptors [59]. These are also likely to be mechanisms of ATRA-caused inhibition of HGF induction. If the latter is the main mechanism, however, there is the question of why cAMP-induced HGF production, which is certainly mediated by CREB, was enhanced rather than inhibited by ATRA. We do not know the reason for this difference. Interestingly, we have recently shown

that interferon-γ enhances cholera toxin- and 8-bromo-cAMP-induced HGF production and inhibits EGF- and PMA-induced HGF production as ATRA did [60]. Transcriptional responses to interferon-γ require STAT1, which also binds CBP/p300 [61,62].

Chattopadhyay et al. [63] have recently reported that HGF secretion and expression in the human malignant glioma cell line U87 are strongly inhibited by ATRA and by 9-*cis*- and 13-*cis*-RAs. HGF and its receptor, c-Met, are expressed at high levels in these cells and exert a strong proliferative action in an autocrine fashion. It is possible that retinoids inhibit HGF production in U87 cells by a mechanism similar to that involved in their inhibition of PMA- and EGF-induced HGF production in human skin fibroblasts.

In summary, this study has demonstrated that ATRA and 9-*cis*- and 13-*cis*-RAs enhanced cholera toxin- and 8-bromo-cAMP-induced HGF production in human skin fibroblasts. In contrast, HGF production induced by EGF or PMA was inhibited by the three retinoids. These results suggest that the retinoids augment the induction of HGF production caused by increased intracellular cAMP.

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